

Voltage-gated sodium channel expressed in cultured human smooth muscle cells: involvement of SCN9A

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Abstract Voltage-gated Na^+ channel (I_{Na}) is expressed under culture conditions in human smooth muscle cells (hSMCs) such as coronary myocytes. The aim of this study is to clarify the physiological, pharmacological and molecular characteristics of I_{Na} expressed in cultured hSMCs obtained from bronchus, main pulmonary and coronary artery. I_{Na} was recorded in these hSMCs and inhibited by tetrodotoxin (TTX) with an IC_{50} value of approximately 10 nM. Reverse transcriptase/polymerase chain reaction (RT-PCR) analysis of mRNA showed the prominent expression of transcripts for SCN9A, which was consistent with the results of real-time quantitative RT-PCR. These results provide novel evidence that TTX-sensitive Na^+ channel expressed in cultured hSMCs is mainly composed of $\text{Na}_v1.7$.

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Keywords: Voltage-gated sodium channel; Cultured human smooth muscle cell; SCN9A; $\text{Na}_v1.7$; RT-PCR; Tetrodotoxin

1. Introduction

The voltage-gated Na^+ channel (I_{Na}) exists in a variety of excitable cells including nerves, heart and skeletal muscle. In response to depolarizing stimuli, the channels open and play an essential role in the rising phase of action potential, which is important for impulse generation and conduction. I_{Na} is an integral membrane protein composed of α and auxiliary β -subunits [1–3]. Several α -subunit Na^+ channel genes have been cloned and functionally analyzed in heterologous expression systems [1,2]. Until now, ten types of α -subunit Na^+ channel genes denoted as SCN1A to SCN11A [1,3] have been identified, and are distributed among mammalian cells.

I_{Na} is also identified in several types of freshly isolated smooth muscle cells (SMCs) including vascular, urinary and gastrointestinal SMCs [4–8]. The jejunal circular human SMCs (hSMCs) express a tetrodotoxin (TTX)-insensitive I_{Na} [9], probably $\text{Na}_v1.5$ known as the Na^+ channel gene in heart. However, I_{Na} expressed in SMCs [4–8] is a TTX-sensitive type, indicating that

different types of α -subunit Na^+ channel genes may be involved. Esophageal hSMCs express TTX-sensitive I_{Na} , $\text{Na}_v1.4$ [10]. Existence of I_{Na} has also been reported in cultured hSMCs such as bronchial (hBSMCs) [11], coronary arterial (hCASMCS) [12,13], pulmonary arterial (hPASMCS) and aortic SMCs [14–16], but the types of I_{Na} expressed in cultured hSMCs remain unknown.

Therefore, we investigated the molecular, pharmacological and physiological characteristics of I_{Na} , expressed under culture conditions in hSMCs. Here, we show that cultured hSMCs possess TTX-sensitive I_{Na} mainly composed of $\text{Na}_v1.7$.

2. Materials and methods

2.1. Cell preparation

Culture cells isolated from normal human bronchus (hBSMCs), main pulmonary artery (hPASMCS) and large coronary artery (hCASMCS) were purchased from Clonetics Corporation (San Diego, USA). The hSMCs were cultured in medium supplemented with 5% fetal calf serum, human epidermal growth factor (0.5 $\mu\text{g}/\text{ml}$), insulin (5 mg/ml), human fibroblast growth factor (1 $\mu\text{g}/\text{ml}$), gentamycin (50 $\mu\text{g}/\text{ml}$), and amphotericin B (0.05 $\mu\text{g}/\text{ml}$) [SmGM-2 Buffer-Kit, Clonetics] in an atmosphere of 5% CO_2 and 95% air at 37 °C. At confluence, the cells were passaged using 0.05% trypsin in 0.02% EDTA, and they were subcultured in the same medium. Cells isolated by trypsin just before confluence at passages 3–7 were used for later experiments. The cells were identified as SMCs by the expression of α -actin as shown previously [17].

2.2. Solutions and drugs

The composition of control Tyrode solution was as follows (in mM): NaCl 136.5, KCl 5.4, CaCl_2 1.8, MgCl_2 0.53, glucose 5.5 and HEPES–NaOH buffer 5.5 (pH 7.4). To block K^+ currents, the patch pipette contained (in mM): CsCl 140, EGTA 10, MgCl_2 2, Na_2ATP 3, guanosine-5'-triphosphate (GTP, sodium salt; Sigma) 0.1, and HEPES–CsOH buffer 5 (pH 7.2). 4-aminopyridine (4-AP, 4 mM), a voltage-dependent K^+ channel blocker, tetraethylammonium (2 mM), a Ca^{2+} -activated K^+ current blocker, and Ba^{2+} (1 mM), a blocker of an inwardly rectifier K^+ current [18], were added to the control Tyrode solution. In addition, nifedipine (1 μM) was included into the bathing solution to block the voltage-dependent L-type Ca^{2+} currents. In the NMDG $^+$ solution, extracellular Na^+ was replaced with equimolar *N*-methyl-D-glucamine (NMDG $^+$). All experiments were performed at room temperature (20–25 °C).

2.3. Recording technique and data analysis

Membrane currents were recorded with tight-seal whole-cell clamp techniques using a patch-clamp amplifier (EPC-7, List Electronics, Darmstadt, Germany) as previously described [19–21].

The steady-state inactivation was estimated using double-pulse protocol. Conditioning voltage pulses (500 ms in duration) of various membrane potentials were applied from a holding potential of –80 mV. At 10 ms after the end of each conditioning pulse, a test pulse of +0 mV

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Abbreviations: I_{Na} , voltage-gated Na^+ channel; SMCs, smooth muscle cells; TTX, tetrodotoxin; RT-PCR, reverse transcriptase/polymerase chain reaction

Table 1
PCR primers used for amplification of voltage-gated Na⁺ channel genes

Gene symbol	Channel	Size (bp)		Sequence (5'–3')
SCN1A	Na _v 1.1	298	Sense	GACAGCATCAGGAGGAAAGG
			Antisense	TGGTCTGACTCAGGTTGCTG
SCN2A	Na _v 1.2	194	Sense	ATCCAGAGGGCTTACAGACG
			Antisense	ATCATACGAGGGTGGAGACG
SCN3A	Na _v 1.3	354	Sense	AATTCTGTGGGGGCTCTAGG
			Antisense	AGCAGCAAGGTTGTCTGAGC
SCN4A	Na _v 1.4	502	Sense	CAGGCATCTTCACAGCAGAG
			Antisense	ACCATGAGGAAGACGGTGAG
SCN5A	Na _v 1.5	618	Sense	ACCATCGTGAACAACAAGAGCC
			Antisense	GGCAGCCAGCTTGACAATACAC
SCN6A	Na _x	449	Sense	AAGAGGTGTCTGGGCAGGAT
			Antisense	GACCAGCATCTGTCTCTGTTG
SCN8A	Na _v 1.6	599	Sense	GAGGTGAAGCCTCTGGATGA
			Antisense	CGGATGGTCTTTCTCTGCTC
SCN9A	Na _v 1.7	403	Sense	GAGGCCTGTTTCACAGATGG
			Antisense	TGGGGCCAAGATCTGAGTAG
SCN10A	Na _v 1.8	453	Sense	CTTGGGCTTTCCTCTCACTG
			Antisense	AGGCGAGGCCIAGAAAAGAC
SCN11A	Na _v 1.9	343	Sense	GGCTGTGCGTTAAGAAGGTC
			Antisense	ACCCTGAGCACTCTGAAGGA

was applied. The ratio of I_{Na} amplitude with and without conditioning pulses was plotted against each conditioning voltage. From current–voltage (I – V) data, the steady-state activation curve was derived by using the following equation: $g_{Na} = I_{Na} / [(V_m - E_{Na})]$, where I_{Na} is the peak current amplitude at each membrane potential (V_m), g_{Na} is the chord conductance, and E_{Na} is the Na⁺-equilibrium potential. E_{Na} was obtained from I – V curve, where the I – V curve crossed over the zero line. The time course of recovery from inactivation was measured by double-pulse protocols. The first (PI, 50 ms) and the second pulse (PII, 50 ms) with variable interpulse intervals were applied from –80 to +0 mV.

Data were expressed as the means \pm S.E. Student's t -test was used for statistical analysis and $P < 0.05$ was significant.

2.4. RNA extraction, RT-PCR and real-time quantitative RT-PCR

Total cellular RNA was extracted using ISOGEN (Nippon Gene, Tokyo). For reverse transcriptase/polymerase chain reaction (RT-PCR), complementary DNA (cDNA) was synthesized from 1 μ g of total RNA with reverse transcriptase with random primers (Toyobo, Osaka) as shown previously [18]. The reaction mixture was then subjected to PCR amplification with specific forward and reverse oligonucleotide primers for 30 cycles consisting of heat denaturation, annealing, and extension. PCR products were size-fractionated on 2% agarose gels and visualized under UV light. Primers were chosen based on the sequence of human SCN1-6A and 8A–11A as shown in Table 1. Table 1 also shows the Na⁺ channel protein created by each SCN gene. Total RNA of human fetal brain, skeletal muscle and heart (Toyobo, Osaka) was used for positive control.

Real-time quantitative RT-PCR was performed with the use of real-time Taq-Man technology and a sequence detector (ABI PRISM® 7000, Applied Biosystems, Foster City, CA) [22]. Gene-specific primers and Taq-Man probes were used to analyze transcript abundance. The 18S ribosomal RNA level was analyzed as an internal control and used to normalize the values for transcript abundance of SCN family genes. We performed six independent experiments.

3. Results

3.1. Voltage-gated Na⁺ channels expressed in cultured hSMCs

Fig. 1A shows a typical recording of I_{Na} expressed in hBSMCs. The cell was held at –80 mV and the command

voltage pulses were applied to various membrane potentials. At potentials more positive than –40 mV, a transient inward current was elicited (Fig. 1A). The current–voltage (I – V) relations measured at the peak of the inward current are shown in B. The peak amplitude of the inward current was observed at approximately –10 mV. The time course of the inactivation of the current was well fitted as a single exponential with τ value of 1.1 ± 0.2 ms ($n = 5$) at +0 mV. Replacement of Na⁺ with NMDG⁺, an impermeable cation, completely abolished the inward current (Fig. 1C). Nifedipine (10 μ M, $n = 3$) did not inhibit it, but TTX (1 μ M) completely blocked it (Fig. 1D). TTX inhibited it concentration-dependently with an IC₅₀ value of 5.9 nM ($n = 5$) in hBSMCs as shown in Fig. 1E. I_{Na} was detected in 38% of the total cells tested ($n = 95$).

Similarly, I_{Na} was recorded in cultured hPASCs (Fig. 2A, 20%, 10 out of 50 cells examined) and hCASCs (Fig. 2B, 15%, 6 out of 40 cells). TTX inhibited I_{Na} expressed in cultured hCASCs with an IC₅₀ value of 6.5 nM ($n = 4$).

3.2. Voltage-dependent characteristics of I_{Na}

Fig. 2C shows typical examples of I_{Na} recordings used to obtain the steady-state inactivation protocol in hBSMCs (see Section 2). The relation between membrane potentials and the I_h value (Fig. 2C) was fitted to the following equation (Boltzmann equation) using the least-squares methods: $I(V)/I_{max} = 1 / \{1 + \exp[(V - V_h)/k]\}$, where V is the membrane potential in mV, V_h is the membrane potential at half maximum, and k is the slope factor. The value of V_h and k was -37 ± 5 mV and 11.6 ± 3 mV ($n = 4$), respectively. The steady-state activation curves were obtained from the conductance (g_{Na}) and also fitted by Boltzmann equation (Fig. 2D). The value of V_h and k was -16 ± 5 and -5.3 ± 1.0 mV ($n = 4$), respectively. The overlap of the steady-state activation and inactivation curve at potentials more positive than –40 mV determines the I_{Na} window currents. Fig. 2E and F show the time courses of recovery from the inactivation of I_{Na} . By fitting

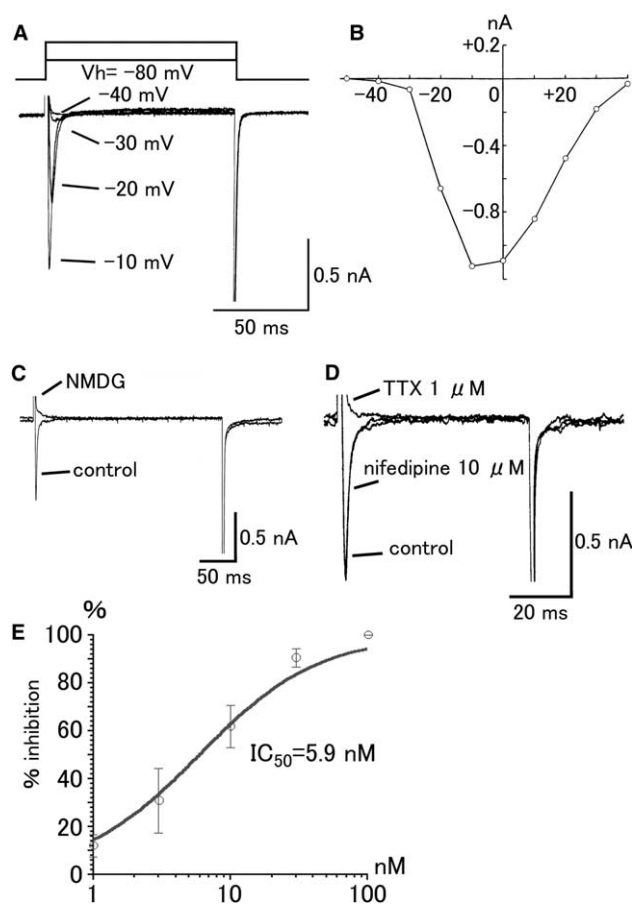


Fig. 1. I_{Na} expressed in cultured human bronchial SMCs. The original current traces elicited by depolarizing pulses are indicated in A. The current–voltage (I – V) relations measured at the peak are illustrated in B. The I – V relations are shown after the leakage currents were subtracted. (C) Effects of replacement of extracellular Na^+ with NMDG $^+$. The current traces in C were elicited from a holding potential of -80 to $+0$ mV. (D) Effects of nifedipine and TTX on the transient inward current. (E) Concentration-dependent inhibition of I_{Na} by TTX. The cells were held at -80 mV, and command voltage-pulses to $+0$ mV (100 ms in duration) were applied at 0.2 Hz. The inhibitory effect of TTX on the current amplitude measured at the peak is plotted against various concentrations of TTX. Data are shown as means \pm S.E. ($n = 5$) and fit by a Michaelis–Menten simple bimolecular model: % inhibition = $100 / \{1 + (IC_{50}/[TTX])\}$, where IC_{50} is 50% inhibitory concentration for TTX. The data were best fit with an IC_{50} value of 5.9 nM.

a single exponential function to the data, the recovery time constants were calculated as 24 ± 5 ms ($n = 4$).

3.3. Expression of voltage-gated Na^+ channel (SCN) in cultured hSMCs

We investigated the expression of SCN channel family members (SCN1A–11A) except SCN7A mRNA in cultured hSMCs, because SCN6A and 7A are probably the same gene [23]. The amplitude of SCN cDNA fragments was of predicted molecular size, identical to cDNA fragments amplified from reversely transcribed mRNA. The transcript of SCN5A was only detected in hPASCs, and that of SCN3A was detected only in hCASCs. The transcripts of SCN1A, 2A, 4A, 6A, 10A, and 11A (data not shown) were not detected in these cells. The transcript of SCN8A was detected weakly in hBSCs and hPASCs. The definite transcript of SCN9A was detected in all of the cells. A positive control for SCN1A–

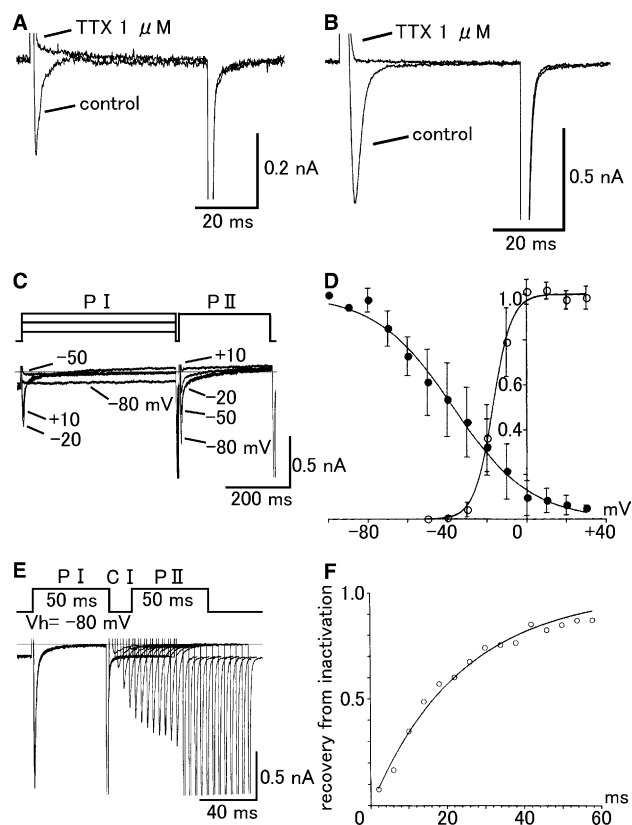


Fig. 2. (A) and (B) I_{Na} expressed in cultured hPASCs (A) and hCASCs (B). The cells were held at -80 mV and command voltage pulses to $+0$ mV were applied. (C) and (D) Steady-state activation and inactivation curves for I_{Na} expressed in hBSCs. The typical original current traces for obtaining the steady-state inactivation curves are indicated in C and the data were fitted by Boltzmann equation (D). In D, the data represent means \pm S.E. value obtained from four different cells. Open circles (steady-state activation curve), closed circles (steady-state inactivation curve). (E) and (F) Recovery from inactivation for I_{Na} . The typical original current traces are shown in E. The data were fitted by single exponential function, where the time constant of recovery from inactivation was 23.9 ms in this cell (F).

3A, SCN4A and SCN5A–6A was observed in human fetal brain, skeletal muscle and heart, respectively.

Expression of SCN channel family member genes (SCN4A–5A, 8A–9A) was also investigated by real-time quantitative RT-PCR. Transcript levels were normalized to 18S ribosomal housekeeping gene. As shown in Fig. 3B, expression levels of SCN9A mRNA were much higher than those of SCN8A. The transcripts of SCN4A and SCN5A were much less than those of SCN9A.

4. Discussion

The present study demonstrated the presence of I_{Na} under culture conditions in hSMCs, which was consistent with the previous papers [11–14]. TTX inhibited it concentration-dependently with an IC_{50} value of approximately 10 nM. These findings indicate that I_{Na} expressed in cultured hSMCs closely resembles TTX-sensitive I_{Na} found in human brain and skeletal muscle, but is different from that found in human heart [1]. Similar TTX-sensitive I_{Na} has been reported in

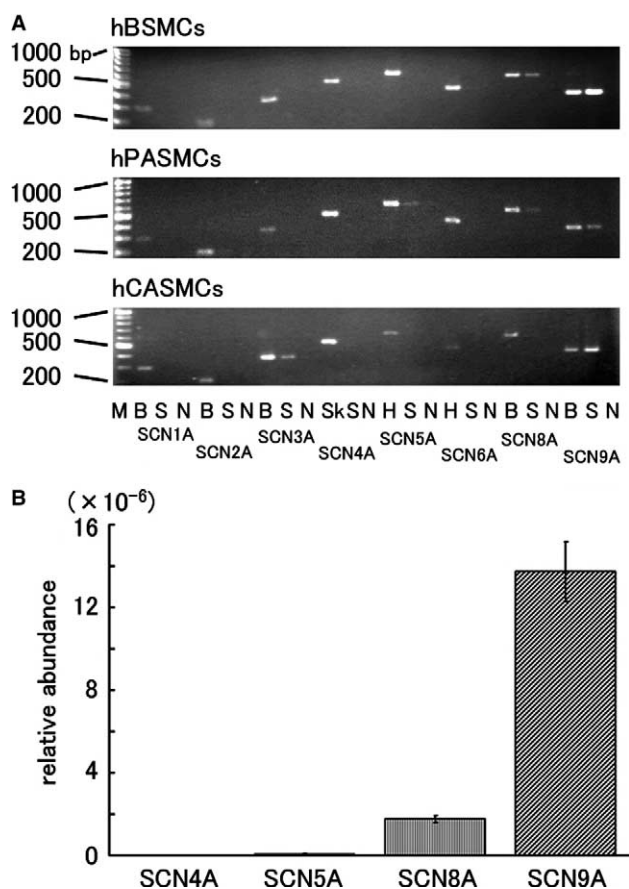


Fig. 3. Expression of I_{Na} genes detected by RT-PCR in cultured hSMCs. (A) RT-PCR. As a positive control, SCN1A–3A were observed in human fetal brain. SCN4A and 5A–6A were observed in human skeletal muscle and human heart, respectively. N, negative control; B, human fetal brain; H, human heart; Sk, human skeletal muscle; S, cultured hSMCs (hBSMCs, hCASCs and hPASCs). (B) Real-time quantitative RT-PCR in hBSMCs. The expression levels of SCN channel genes were normalized to those of the 18S ribosomal RNA levels. Data are means \pm S.E. from six independent samples.

several types of freshly isolated SMCs [4–8]. Recently, using molecular techniques, human jejunal circular and esophageal SMCs have been shown to express a TTX-insensitive I_{Na} gene, SCN5A [9], and a TTX-sensitive Na^+ channel gene, SCN4A [10], respectively. In the present studies using RT-PCR, SCN5A was detected in hPASCs. However, it is unlikely that $Na_v1.5$ contributes to form I_{Na} in these cells because low concentration of TTX could completely inhibit I_{Na} . And, SCN4A did not exist. Alternatively, the prominent expression of SCN9A was detected in all types of cultured hSMCs examined. TTX-sensitivity of Na^+ channels is determined by whether one amino acid in SS2 regions of repeat I is aromatic or not, and the amino acid (Y374) is aromatic, suggesting that $Na_v1.7$ belongs to TTX-sensitive I_{Na} [24]. Thus, these results suggest that $Na_v1.7$ is mainly responsible for I_{Na} expressed in cultured hSMCs, which was consistent with the results of real-time quantitative RT-PCR analysis.

I_{Na} expressed in cultured hSMCs was activated at potentials greater than -40 mV and displayed a half inactivation voltage (V_h) of approximately -37 mV, and the window current was observed at potentials more positive than -40 mV. During the repolarization period of 10 ms used in our protocol, parts of I_{Na} can recover, and the residual current may affect the inactivation

curves. But, it is negligible at potentials negative than -30 mV due to the small amplitude of I_{Na} . Thus, these values are more positive than those reported for I_{Na} reported in muscle, nerves [23,25], and freshly isolated SMCs [7–9], proposing that it has atypical characteristics of I_{Na} . But, since the cultured SMCs used here had a membrane potential of approximately -40 mV as described previously [18], it is reasonable that I_{Na} expressed in cultured hSMCs contributes to form membrane potentials and muscle excitability in these cells. In addition, it is likely that small depolarizing stimuli open I_{Na} furthermore, thereby inducing an increase of Na influx and intracellular Na^+ concentration ($[Na^+]_i$). The increase in $[Na^+]_i$ alters the driving force for Na^+ , the rate of movement of Na^+ ions by the Na^+/K^+ pump and then the Na^+/Ca^{2+} exchanger, which may subsequently raise intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in these cells.

Expression of $Na_v1.7$ has been found at high levels in peripheral nervous systems and neuroendocrine cells [26,27], which is involved in cell excitability and secretion. Recently, it has been reported in rat and human prostate cancer cell lines, pheochromocytoma and thyroid gland tumor [28]. Physical significance of I_{Na} expressed in these tumor cells remains unclear, but it may be related to the tumor invasion, metastasis and cell proliferation. I_{Na} has been identified in various types of phasic SMCs [8], which usually generate action potential. On the other hand, it is not expressed in freshly isolated tonic SMCs such as human coronary artery and bronchial muscle [11–13], in contrast to the cultured cells [11–14], though expression of I_{Na} has been reported only in rabbit pulmonary arterial cells [6]. Thus, it is likely that I_{Na} expressed in cultured hSMCs is limited to the culture conditions, where cellular dedifferentiation and proliferation may be involved [13,15]. Expression of I_{Na} could be found in parts of the cells examined in this study, which may also support this notion. However, these changes may be occurred under various pathophysiological conditions such as vascular injury, atherosclerosis and asthma [29–32]. Therefore, the possible expression of I_{Na} under these conditions is worth considering, and further studies are necessary to clarify the physiological significance of the channel.

In conclusion, the present study provides novel evidence that TTX-sensitive Na^+ channel is expressed under culture conditions in hSMCs, mainly composed of $Na_v1.7$.

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